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Dose-related effect of acetylsalicylic acid on replication of varicella zoster virus *in vitro*

(increased yields/plaque inhibition/Reye syndrome)

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ABSTRACT **Cultivation of human embryonic lung (HEL) cells in media containing acetylsalicylic acid (ASA) at 100 μg/ml and maintenance at this level after inoculation with either cell-free varicella zoster virus (VZV) or virus-infected cells resulted in a 2- to 4-fold increase in yields of cell-free virus released by sonication. The degree of enhancement was dependent upon multiplicity of infection and time of harvest. Enhanced viral yields were not consistently accompanied by an increase in the number of infected cells, nor was VZV plaque formation in HEL indicator cells significantly increased in the presence of ASA at 100 μg/ml. In the presence of ASA at 500–1000 μg/ml, VZV plaque formation was inhibited; this inhibition was partially reversible, depending on concentration and period of exposure to ASA. These findings may bear on the apparent association between ASA ingestion and the development of Reye syndrome after infection with varicella virus.**

Varicella zoster virus (VZV) was isolated in 1953 (1). Yet, the pathogenesis of the two diseases—commonly called chickenpox and shingles—produced by this ubiquitous virus remains obscure. When cultivated *in vitro*, VZV is uniquely cell associated; cell-free virus can be released only by special treatment such as sonication of the infected cells, and then in relatively small amounts. In spite of these constraints, interest in VZV increases, as recently reviewed (2). Infection is recognized as potentially life threatening in the immunocompromised human host. An attack of varicella or of influenza is usually antecedent to the onset of Reye syndrome (RS) of which some 600 to 1200 cases occur annually in the United States, with a mortality of 20–30% (3). The syndrome, usually seen in children, presents as an acute, noninflammatory encephalopathy with cerebral edema and fatty metamorphosis of the liver. Although the etiology of RS remains unknown, epidemiologic studies suggest that the administration of acetylsalicyclic acid (ASA) during the prodromal viral illness increases the risk of development of RS (4–6). While the association has been questioned, governmental agencies have advised against the use of salicylate-containing medications for children with influenza or chickenpox (3). In this communication we report evidence documenting a dose-related enhancing or inhibitory effect of ASA on yields of VZV propagated *in vitro*.

METHODS AND MATERIALS

Tissue Culture and Virus. Two lines of human embryonic lung (HEL) fibroblast cultures were prepared and maintained on Eagle's minimal essential medium supplemented with 2% fetal calf serum, penicillin at 100 units/ml, streptomycin at 100 μg/ml, or gentamicin at 100 μg/ml. One line was used in the 4th to the 10th passage and the other in the 22nd to the 39th passage. The strain of VZV was isolated from a patient with varicella and used after 19–55 cultural passages. Stock virus was prepared and cell-free virus was concentrated with polyethylene glycol 6000 as previously described (7).

Harvest of Virus. Virus-infected cells were harvested by washing infected cultures maintained in 25-cm^2 Corning flasks with phosphate-buffered saline and incubating for 5–10 min with 1.0 ml of 0.25% trypsin in phosphate-buffered saline at 37°C; the detached cells were then transferred to 10% fetal calf serum in phosphate-buffered saline to inactivate residual trypsin, centrifuged at 180 × *g* for 10 min, resuspended in 1.0 ml of Eagle's minimal essential medium, and kept at 4°C.

Cell-free virus was obtained from three cultural sources: (*i*) *Growth medium.* Five milliliters of growth medium was removed from infected cultures maintained in 25-cm^2 flasks and centrifuged at 400 × *g* for 15 min, and the supernatant was kept at 4°C. (*ii*) *Glass bead treatment.* Infected cells were harvested mechanically by addition of 10–15 glass beads (2–3 mm in diameter) and 0.5 ml of SPGA medium (8) to each flask; after gentle shaking the freed cells were removed and the flask was rinsed twice with 0.5 ml of SPGA; the cells and washings were centrifuged at 600 × *g* for 15 min, and the supernatant containing virus was kept at 4°C. (*iii*) *Sonicated infected cells.* The cellular sediment after centrifugation was resuspended in 1.0 ml of SPGA, sonicated in a 40-ml conical centrifuge tube with the microtip of a model W 200 Heat Systems sonicator (60% power, 10–15 sec), and centrifuged at 600 × *g* for 15 min, and the supernatant fluid was kept at 4°C. The total amount of cell-free virus mechanically released per culture was expressed as plaque-forming units (pfu) by combining the results after titration of supernatants *ii* and *iii*—i.e., after glass bead treatment and sonication.

Virus Titration. Preliminary studies confirmed the findings of other workers that a semisolid overlay was not necessary for assay by plaque counts (8, 9). Serial 1:10 dilutions of centrifuged supernatant fluid or of trypsin-treated infected cell suspensions were prepared in Eagle's minimal essential medium. Duplicate HEL monolayer cultures in 35-mm plates were inoculated with 0.2 ml per diluted sample, incubated for 1.5–2 hr at 37°C in a 5% CO_2/air atmosphere, washed once, and refed with 2.0 ml of Eagle's minimal essential medium containing 2% fetal calf serum, 0.225 M sodium bicarbonate, and gentamicin at 100 μg/ml. Plaques were counted on the fifth or sixth day with the aid of an inverted microscope.

Incorporation of Salicylate Compounds into the Culture System. ASA and the sodium salt of salicylic acid were obtained from Sigma; stock solutions (10 mg/ml) of each were prepared in water and warmed before use. For studies on viral yields ASA was added in 100 μg/ml amounts to 25-cm^2

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Abbreviations: ASA, acetylsalicylic acid; E.I., enhancement index; VZV, varicella zoster virus; HEL cells, human embryonic lung cells; RS, Reye syndrome; moi, multiplicity of infection; pfu, plaque-forming units.

flasks containing freshly trypsin-treated HEL cells suspended in Eagle's minimal essential medium/Hanks' salt solution supplemented with 15% fetal calf serum and penicillin/streptomycin or gentamicin. After 48–72 hr the medium was aspirated, and cultures were inoculated with 0.5 ml of polyethylene glycol-concentrated cell-free VZV or a suspension of infected cells; dilutions of ASA in medium were added, and cultures were agitated gently for 2 hr at 37°C, aspirated, and fed with 5.0 ml of Eagle's minimal essential medium/fetal calf serum containing ASA. Control cultures received equivalent volumes of phosphate-buffered saline.

For assessment of the influence of ASA on the number of virus-infected cells or on the amount of cell-free virus harvested from infected flask cultures, an enhancement index (E.I.) (10, 11) was calculated at various stages of the growth cycle. The average number of pfu developing in paired monolayer plate cultures inoculated with serially diluted harvests from ASA-treated flask cultures was divided by the average number of pfu developing in paired cultures inoculated with materials from ASA-free control flasks. In some experiments, paired flasks were harvested, and the E.I. was then derived from the average pfu developing in four culture plates. Results from control cultures were assigned an E.I. of 1.0 (11); an E.I. of 2 or greater was considered positive (10).

To determine the effect of ASA on plaque formation, monolayer cultures were established in 35-mm plates in the presence of ASA at 100 μg/ml; after 48–72 hr, media were aspirated, and cultures were inoculated with 0.2 ml of cell-free virus or an infected cell suspension and allowed to adsorb 1.5–2 hr at 37°C. ASA (100 μg/ml) was maintained in the medium throughout in some cultures, and in others was added only after adsorption. Infected control cultures were handled similarly with comparable additions of phosphate-buffered saline. For maintenance of infected cultures in higher concentrations of ASA and ASA-related compounds, freshly prepared drug-containing medium was added after adsorption and changed every 3 or 4 days; when ASA was removed from the system, cultures were washed three times with Eagle's minimal essential medium before addition of ASA-free medium.

RESULTS

Preliminary experiments indicated that ASA at concentrations of 50–100 μg/ml (0.277–0.555 mM) did not change the pH of the medium (phenol red), and when ASA was introduced into freshly prepared cell suspensions and thereafter incorporated in the culture medium, it produced no overt adverse effect on attachment and replication of HEL cells. In five experiments the mean number of viable cells obtained on trypsinization of cultures maintained in ASA at 100 μg/ml for 48 hr was 6.1×10^5 compared with 6.8×10^5 cells from untreated cultures. However, when ASA was added at 500 μg/ml the pH was lowered, and after 48 hr the number of viable cells was slightly reduced. When 1000 μg/ml was added to the cell suspension, confluent monolayer cultures were not obtained.

Yields of Cell-Free VZV After Use of Polyethylene Glycol-Concentrated Cell-Free Viral Inocula. Cultures established and maintained in ASA at 50 or 100 μg/ml were inoculated with polyethylene glycol-concentrated cell-free virus and harvested mechanically 46–48 hr later or at 72–80 hr. Cell-free viral yields were compared with those of control cultures maintained in parallel. As shown in Table 1, the 2- to 4-fold enhancement of cell-free virus detected in ASA-maintained cultures was time dependent and related to the multiplicity of infection (moi). In cultures inoculated with a relatively low moi (approximately 0.01), enhancement was observed at 72–80 hr, while with a moi of approximately 0.1, enhancement occurred at 46 hr in the presence of ASA at 50 or 100 μg/ml. With the higher moi, the peak yield of cell-free virus was recorded at 46 hr in ASA-maintained cultures and at 78 hr in control cultures. In two experiments small amounts of cell-free virus were spontaneously released into the growth medium at 72 hr in both ASA and control cultures, with yields slightly higher in the ASA-maintained cultures (15–58 pfu/ml as compared to 4–8 pfu/ml).

Table 1. Effect of ASA on yields of cell-free virus after inoculation of pretreated HEL cultures with polyethylene glycol-concentrated cell-free VZV

Exp.	moi	ASA, μg/ml	Harvest time, hr	pfu × 10^{-2} per culture: Control	pfu × 10^{-2} per culture: ASA-treated	EI
1	0.01	100	48	0.3	0.4	1.3
		100	80	17.0	52.0	3.1
2	0.01	100	48	0.4	0.4	1.0
		100	72	6.0	29.0	4.8
3a	0.1	50	46	3.7	15.0	4.1
		50	78	4.8	2.8	0.6
3b	0.1	100	46	3.7	7.9	2.1
		100	78	4.8	3.3	0.7

Yields of Cell-Free VZV After Use of Cellular Inocula. Cultures were established with ASA at 100 μg/ml and inoculated 48–72 hr later with VZV-infected cells at various moi ranging from approximately 0.01 to 1.0. At intervals, cultures were harvested mechanically and cell-free viral yields from ASA-maintained cultures were compared with yields from cultures maintained without ASA. The E.I. values obtained in 11 experiments (10 performed in duplicate) are indicated in Fig. 1 by solid circles. A 2- to 4-fold increase in yields of cell-free virus was detected in all of 8 ASA-maintained cultures harvested between 40 and 65 hr after inoculation. Cell-free viral yields from ASA-maintained cultures ranged from a low of 10 pfu early in the growth cycle to 4.8 ×

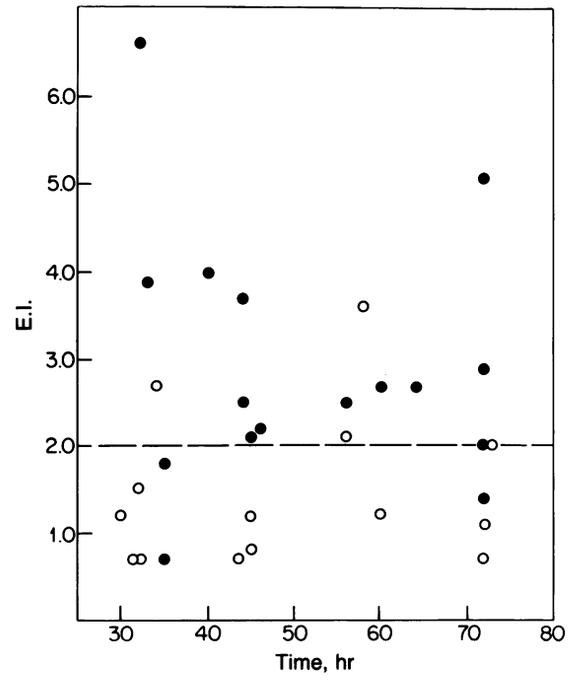

FIG. 1. Comparison of yields of VZV (●, cell-free virus; ○, infected cells) from cultures maintained with and without ASA at 100 μg/ml after inoculation with infected cells. An E.I. of 2.0 or greater, indicated by the broken line, was considered positive.

10^3 pfu at a later period; these compared with 14 pfu and 1.8 $\times$ 10^3 pfu, respectively, in control cultures. Peak yields of cell-free virus occurred concurrently in control and ASA-maintained cultures in 3 of 4 experiments in which cultures were harvested at more than one interval during the replication cycle.

Yields of Infected Cells After Use of Cellular Inocula. Cultures were established with ASA at 100 μg/ml and inoculated after 48–72 hr with VZV-infected cells; at intervals cultures were harvested by trypsinization, cell suspensions were assayed for infectivity, and resulting titers were compared with yields from cultures maintained without ASA. The E.I. obtained from 6 experiments (5 performed in duplicate) are indicated in Fig. 1 by open circles. A 2- to 3-fold increase in the number of infected cells was observed in 3 of 9 cultures harvested between 40 and 72 hr. Counts of infected cells in ASA-maintained cultures ranged from a low of 1.3 $\times$ 10^3 pfu early in the growth cycle to a high of 3.5 $\times$ 10^5 pfu later on, and from 2.0 $\times$ 10^3 pfu to 3.0 $\times$ 10^5 pfu in control cultures; in both sets of cultures peak yields occurred concurrently.

Two of the cultures showing a significant increase on assay of infected cells (56 and 72 hr, Fig. 1) were from the same experiment in which cell-free virus from cohort duplicate cultures was also assayed. Comparison of cell-free yields at 32, 56, and 72 hr resulted in E.I. values of 6.6, 2.5, and 1.5, respectively, whereas corresponding indices of infected cells were 1.5, 2.1, and 2.0, respectively. In another experiment (also summarized in Fig. 1), cell-free viral yields in ASA-treated cultures at 44 and 72 hr showed a 2.5- to 5-fold increase, while no increase in number of infected cells was observed in cohort cultures (E.I. of 0.7 and 1.0, respectively).

Influence of ASA on VZV Plaque Formation. If infected cells in the presence of ASA are more fragile or sensitive to the action of trypsin, such cells might be destroyed during trypsinization and resuspension. Experiments, therefore, were performed to determine if ASA *per se* influenced the sensitivity of monolayer cultures as indicators of VZV. Dilutions of cell-free virus or infected cells were added to HEL plate cultures either nontreated or pretreated with ASA at 100 μg/ml; after 5 days of incubation in ASA-containing or drug-free medium, VZV plaques were counted. As shown in Table 2, no significant difference in the number of plaques was found. A slight increase in the mean number of pfu was observed in pretreated indicator cultures inoculated with VZV-infected cells, but enhancement was inconsistent and less than 2-fold in three of four experiments.

However, additional experiments showed that plaque formation was inhibited in the presence of concentrations of ASA greater than 100 μg/ml. In cultures inoculated with cell-free VZV and maintained with ASA at 500 μg/ml for 5 days, fewer and smaller plaques developed. Table 3 summarizes the results of three experiments, in which the mean

Table 2. VZV plaque formation after pretreatment and maintenance of indicator HEL cells with ASA at 100 μg/ml

ASA addition	Mean pfu per culture			
	Cellular inoculum		Cell-free inoculum	
	Non-treated	ASA-treated	Non-treated	ASA-treated
After viral adsorption*	25	23	27	27
When culture established and thereafter†	25	38	35	31

*Two experiments, each with cultures in duplicate.
†Four experiments, each with cultures in duplicate.

Table 3. Effect of ASA at 500 and 1000 μg/ml on VZV plaque formation after inoculation of HEL indicator cultures with cell-free VZV

ASA, μg/ml	Mean pfu per culture			
	Control	ASA present		After 5–10 days in drug-free medium
		5 days	12 days	
500*	37	5	NT	≥37
1000*	38	0	NT	10
1000†	47	0	0	3

NT, not tested.
*Three experiments, each with cultures in duplicate.
†Two experiments, each with cultures in duplicate.

number of plaques present after 5 days represented only 13.5% of input virus. At 500 μg/ml, inhibition was reversible, for when ASA was removed, new plaques developed with approximately 100% recovery of the input virus after an additional 5–7 days of cultivation in drug-free medium.

The addition of ASA to established HEL cell monolayers at 100 or 500 μg/ml produced no overt morphologic differences. However, when ASA was incorporated in the medium of established cultures at 1000 μg/ml, the fibroblastic pattern altered to an irregular patchy network with small intervening acellular areas. When medium containing ASA at 1000 μg/ml was replaced after 5 days by drug-free medium, the monolayer eventually regained a healthy appearance. In the presence of ASA at 1000 μg/ml for 5 days, plaque formation was completely inhibited after inoculation of cell-free virus (Table 3); thereafter when ASA was removed a reduced number of foci appeared, with an average of 26% recovery of input virus (range of 4–50% recovery in three replicate experiments). Even in cultures maintained for 12 days after inoculation in the presence of ASA at 1000 μg/ml, a few plaques representing 4–8% of the input virus developed within 5–10 days after a shift to ASA-free medium. Plaque formation in cultures inoculated with infected cells was also partially inhibited by incubation with ASA at 500 μg/ml and was completely inhibited by ASA at 1000 μg/ml. Sodium salicylate showed a similar but less marked effect over a 5-day period. In one experiment, 500 μg/ml gave a 38% plaque reduction, and in two experiments 1000 μg/ml yielded a mean reduction of 93% (data not shown).

DISCUSSION

The data demonstrate a biphasic effect of ASA on yields of VZV propagated *in vitro*. Incorporation of 100 μg/ml into the culture medium enhanced cell-free yields, while 5- to 10-fold higher concentrations reduced or inhibited VZV plaque formation, resulting in establishment of a persistent viral infection. Pretreatment of cultures with concentrations of ASA equivalent to 0.555 mM (100 μg/ml) prior to inoculation and its presence thereafter resulted in 2- to 4-fold enhanced yields of cell-free virus as recovered after mechanical harvest and sonication of infected HEL cells. Detection of increased cell-free virus was dependent upon time of harvest and was directly related to the moi. In general, when significant enhancement associated with a relatively high moi was found early in the observation period, this later disappeared. Higher yields of cell-free virus were not paralleled by a consistent increase in the number of trypsinized VZV-infected cells. All of the ASA-treated cultures (8/8) examined between 40 and 65 hr after inoculation showed enhanced yields of cell-free virus, while a significant increase in the number of infected cells was found in only 33% of the cultures (2/6) examined at the same time. If ASA-treated infected cells exhibit increased sensitivity to the action of trypsin, then infected cells might be differentially lost in the pres-

ence of ASA. However, examination of the influence of ASA at 100 μg/ml on viral cell-to-cell spread demonstrated that VZV plaque formation was not significantly altered.

These observations suggest that ASA may act intracellularly, by protecting viral particles from degradation or by increasing the number of viral particles per cell. The cell-associated nature of VZV growth *in vitro* has hampered varicella research for decades. One hypothesis for the low yield of cell-free VZV *in vitro* is that, unlike herpes simplex or cytomegalovirus, intracellular VZV particles are susceptible to lysosomal enzymatic degradation (12). Ultrastructural evidence indicates that VZV particles are degraded intracytoplasmically (13); histochemical studies have demonstrated the presence of acid phosphatase activity and VZV particles in the same cytoplasmic vacuole (14). At 0.25 to 0.5 mM, ASA significantly reduces release of acid phosphatase from rat retinal lysosomes *in vitro* (15). The demonstrated enhanced yields of cell-free VZV from cultured HEL cells in the presence of 0.555 mM ASA may reflect reduced activity of lysosomal VZV-labilizing enzymes, resulting in the preservation of a greater number of infective viral particles per cell.

ASA in excess of 100 μg/ml in the medium inhibited replication of VZV. The inhibitory effect was virustatic, since removal of ASA was followed by the development of focal lesions, with the number of plaques inversely proportional to drug concentration as well as to the length of time the cultures were exposed to ASA. Inhibition of VZV replication at these concentrations is not surprising, since 1–2 mM salicylate abolishes aerobic synthesis of adenosine triphosphate in mitochondria (16). Clinically effective salicylate concentrations in serum range from 0.5 to 3 mM [7–40 mg/dl (16)]. Thus, the observed dose-related enhancing and inhibitory effect of ASA on VZV growth *in vitro* occurred at concentrations comparable to those achieved therapeutically *in vivo*.

While pathological findings in RS developing after an attack of varicella do not provide evidence of VZV activity, our findings may be relevant in explaining an association with the use of ASA. At low therapeutic levels, degradation of intracellular viral particles may be inhibited. At higher levels of ASA, a persistent but non-productive infection might eventuate, with viral containment further potentiated by humoral and cellular immune processes developing in the interim. Eventually, as ASA levels fell, the sensitized patient might exhibit an enhanced and deleterious immunologic response to the release of VZV-specified antigenic materials in the absence of evidence of overt VZV activity.

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